DIGITAL DROPLET PCR ON CHIP FOR QUANTITATIVE ASSESSMENT OF MICRORNAs

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ABSTRACT

In this paper, the layout, fabrication and preliminary testing of a microfluidic chip for the quantitative detection of microRNA (miRNA) molecules is described. The chip integrates the following steps for miRNA quantification: miRNA release, reverse transcription and digital droplet PCR. The experiments for quantifying let-7a miRNA copy numbers were performed using MCF-7 cells. Results show that the digital on-chip and analog off-chip quantifications are in good agreement; the determined concentration on the same sample was 780 templates/μL and 900 template/μl, respectively.

KEYWORDS: miRNA, digital droplet PCR, lab-on-chip

INTRODUCTION

miRNAs, a class of endogenous short non-coding RNAs, play a critical role in various cellular, developmental and physiological processes by means of post-transcriptional regulation of gene expression. Changes in the expression patterns of miRNAs are associated with different diseases, such as cancer, diabetes and muscle disorders [1]. As these regulators are detectable in peripheral blood, they are ideal candidates for blood-based biomarkers. Therefore, it is of great interest to implement the detection of miRNA into a microfluidic device (chip) to allow fast, cheap and accurate quantification. Digital droplet PCR (ddPCR) combines these characteristics and additionally improves reproducibility compared to regular qPCR [2].

EXPERIMENTAL

Fabrication: To automate miRNA analysis, Si chips were fabricated using a microfluidic platform developed at Imec [3]. A photograph and a schematic of the chip are shown in Fig. 1. Fluidic structures (in violet in Fig. 1b) are etched from the front-side of the silicon wafer to a depth of about 50 μm, then a Pyrex wafer is anodically bonded to silicon to provide optical access and fluidic sealing. Finally, a last etch (in green in Fig. 1b) is performed from the backside to the full depth of the silicon wafer; it defines trenches for thermal insulation of the PCR reactor from the remaining chip and holes for fluidic access to the front-side structures. While mixers, T-shaped droplet generators and thermally insulated PCR reactors were included in the chip design, an external thermoelectric Peltier element was used to control the temperature of the reactors up to 100 °C [4]. The chip integrates all steps necessary for miRNA quantification: miRNA release, reverse transcription (RT) and digital droplet PCR (ddPCR).

Materials and methods: To demonstrate on chip the full miRNA detection protocol, let-7a miRNA released from MCF-7 cells was used as a case study. All steps were first tested and optimized off-chip with conventional bench-top tools. The full protocol was then transferred on-chip. In these preliminary experiments, not all characteristics of the chip were exploited; therefore reactions were performed on chip but in order to simplify fluidic handling, mixing was performed off-chip. The first step considers the release of miRNA from cells with cell concentrations in the sample ranging between 1.6 cells/ μl and 2500 cells/ μl. Chemical (Taqman miRNA cells-to-CT kit, Ambion) and thermal lysis (5' at 95°C, in the presence of an RNAse inhibitor, RNaseOut, Life Technologies and performed on the Veriti Thermal Cycler, Applied Biosystems) were compared off-chip. Thermal lysis yielded superior results (as will be discussed later), so only this protocol was transferred on-chip. As a second step, RT was performed on- and off-chip (Veriti Thermal Cycler, Applied Biosystems) using the lysates produced on-chip. Cell lysate was mixed in a 1:2

ratio with RT enzymes, buffer (Taqman miRNA reverse transcription kit, Applied Biosystems) and stem-loop primers (hsa-Let-7a, Applied Biosystems) to generate cDNA (30' at 16°C, 30' at 42°C and 5' at 85°C). In the final step, qPCR mix (ddPCR supermix, Biorad and hsa-Let-7a, Applied Biosystems) was mixed with let-7a cDNA, which had been fully generated on-chip. Part of this solution was used for ddPCR and the other part for analog qPCR (LightCycler 480, Roche) to quantify the number of let-7a molecules present in the sample. To this end, Poisson statistics was used in the analysis of the ddPCR result and a standard curve (performed off-chip) was used for analog qPCR by making use of a dilution series of known let-7a miRNA copies. For the ddPCR experiments, the qPCR mix was partitioned using the T-shaped droplet generator, which enabled the generation of aqueous qPCR mix droplets in oil (Biorad). The droplets were subjected to a heat-activation step for the polymerase (5' at 95°C) and to at least 50 PCR cycles (15s at 95°C, 45s at 60°C).

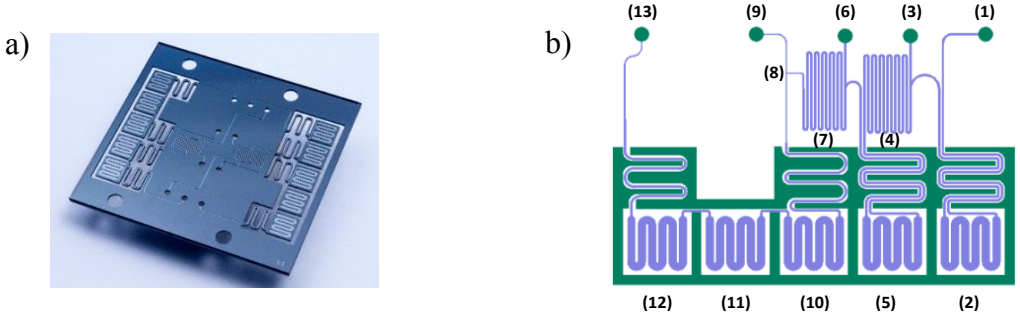

Figure 1: a) Photograph of the chip for miRNA extraction and quantification. b) Schematic of the chip. Cells are loaded from inlet (1) in a microreactor (2) where thermal lysis occurs. RT reagents are loaded from inlet (3), mixed with the lysate in mixer (4) and transferred in the RT microreactor (5) where transcription of one or multiple miRNA fragments occurs. PCR reagents are introduced from the inlet (6). These reagents mix with cDNA in mixer 7 and droplets of the mixture are formed in oil at the T-junction droplet generator (8). Droplets are stored in the three thermal reactors (10, 11, and 12) where PCR is performed.

RESULTS AND DISCUSSION

All previously described steps (lysis, RT and Let-7a detection) were performed separately on-chip as well as off-chip to compare performances. Firstly, we compared off-chip thermal and chemical lysis. Results are shown in Fig. 2a, where the C_t value (measured after lysis, reverse transcription and qPCR), is reported as a function of cell concentration in the starting sample for the two methods. The C_t value decreases steadily when the density of cells increases and saturates when cell density is about 2500 cells/µl in the case of thermal lysis and about 150 cells/µl in the case of chemical lysis. This difference in performance could be attributed to the buffers used for chemical lysis, which could inhibit downstream reaction compared to the nuclease-free water used to perform the heat lysis. Hence, thermal lysis is implemented on-chip.

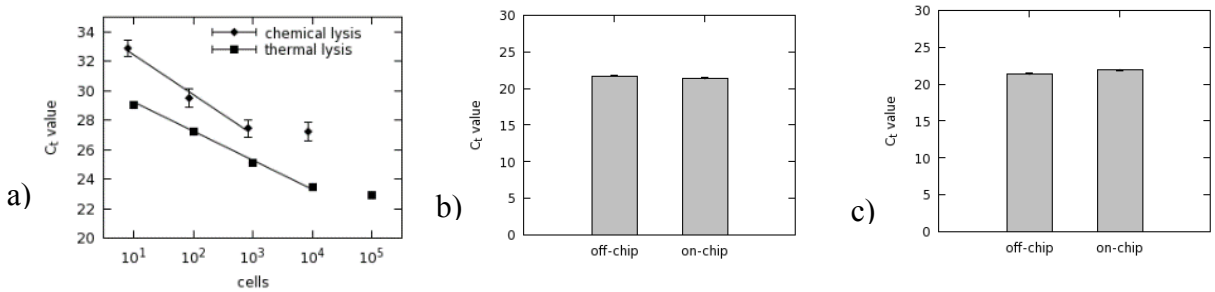

Figure 2 Off- and on-chip comparison for the lysis and RT steps. (a) Threshold cycle values were plotted versus cell concentrations, square: thermal lysis, diamond: chemical lysis. (b) Threshold cycle values for samples lysed off- and on-chip were compared. (c) Threshold cycle values for on-chip lysed samples when RT is performed off-chip and on-chip were compared.

Furthermore, experiments revealed a difference in threshold cycle (C_t) between on and off-chip thermal lysis of only 0.5 as shown in Fig. 2b when both the RT and qPCR steps are performed off-chip.
Secondly, RT was performed on and off-chip using on-chip lysed cells. The performance of the RT step was also evaluated by qPCR. The difference in C_t here was again limited to ~0.5 (see Fig. 2c).
In a final experiment, both lysis and RT steps were performed on-chip and quantification was obtained both on-chip by ddPCR and off-chip by analog qPCR. Fig. 3a shows the result for ddPCR, after conventional analysis; a concentration of ~780 copies/µl was determined. The standard curve shown in Fig. 3b was used to quantify the let-7a concentration. The qPCR result was obtained using the same sample as for ddPCR and is shown in Fig. 3c, which gives a C_t value of 25.9, corresponding to ~900 copies/µl. This demonstrates that concentrations obtained using different techniques are in good agreement.

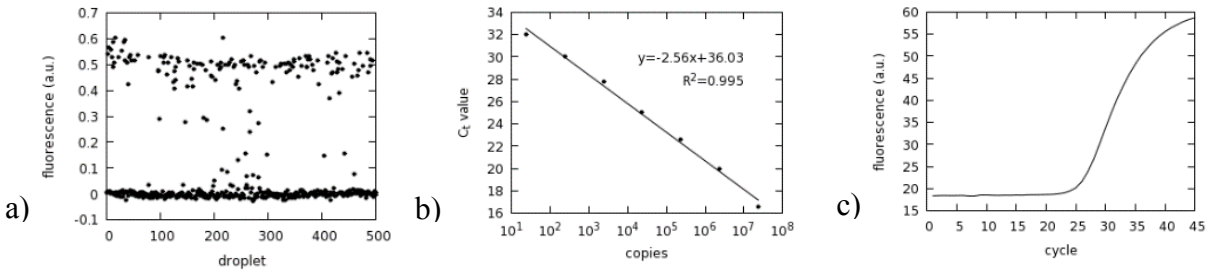

Figure 3: Quantification of let-7a by ddPCR (on-chip) and analog qPCR (off-chip) (a) ddPCR result is shown by plotting the fluorescence intensity for each droplet. The percentage of bright droplets is 25% and corresponds to a concentration of 780 copies/µl. (b) A standard curve performed off-chip is shown for let-7a miRNA. (c) The qPCR curve obtained off-chip using the same sample as for ddPCR. From the C_t value, a concentration of 900 copies/µl is determined using the standard curve (b).

Above results prove that the full assay for miRNA detection can be implemented on a Si-microfluidic platform.

CONCLUSION

In conclusion a microfluidic chip for miRNA quantification has been successfully developed. Initial characterization studies indicate comparable quantification with bench-top tools. More efforts are ongoing to optimize the current system by performing all steps sequentially on chip, decreasing the limit of detection as well as implementing multiplexing capabilities to detect several miRNAs simultaneously.
For future prospective, a further simplification in both chip design and operation can be achieved by using one-step RT-qPCR, which allows the last two steps to be performed in droplets.
Also, as a proof-of-concept, miRNA was extracted from MCF-7 cells, but it is of greater interest to validate the system using clinical blood samples. This adds an additional challenge as a preceding purification step is almost certainly needed.

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